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bcl-2 Inhibits Multiple Forms of Apoptosis but Not Negative Selection in Thymocytes

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Summary

The vast majority of cortical thymocytes die during T cell development while those that survive this selective process accumulate in the medulla. bcl-2, an inner mitochondrial membrane protein, has been shown to inhibit apoptosis in certain cell lines. In the thymus, bcl-2 is regionally localized to the mature T cells of the medulla. To assess the role of bcl-2 in the programmed death of thymocytes, we generated transgenic mice that redirected bcl-2 expression to cortical thymocytes. bcl-2 protected immature $CD4^+8^+$ thymocytes from glucocorticoid, radiation, and anti-CD3-induced apoptosis. Moreover, bcl-2 altered T cell maturation, resulting in increased percentages of $CD3^{hi}$ and $CD4^-8^+$ thymocytes. Despite this, clonal deletion of T cells that recognize endogenous superantigens still occurred. This transgenic model indicates that multiple death pathways operate within the thymus that can be distinguished by their dependence on bcl-2.

Introduction

T cell development involves a selective process that operates in a specialized lymphoid organ, the thymus. The majority of thymocytes (~80%–85%) are immature, coexpress both the CD4 and CD8 accessory molecules, and reside in the cortex of the thymus (reviewed in Fowlkes and Pardoll, 1989; von Boehmer, 1990). Many of these cells express low levels of the CD3–T cell receptor (TCR) complex. In contrast, mature thymocytes (~15%) that are single positive $CD4^+8^-$ or $CD4^-8^+$, and $CD3\text{–}TCR^{hi}$ populate the medulla. A variety of in vivo studies examining the kinetics of $CD4^+8^+$ thymocytes indicates that the vast majority of these thymocytes is destined to die (Penit, 1986; Egerton et al., 1990). These immature $CD4^+8^+$ thymocytes are extremely vulnerable, being highly sensitive to glucocorticoid (Blomgren and Andersson, 1971; Cohen and Duke, 1984), radiation (Sellins and Cohen, 1987), and anti-CD3/TCR-induced (Smith et al., 1989; Shi et al., 1991) apoptotic cell death. The minority of $CD4^+8^+$ thymocytes that survive are presumed to be the precursors of the mature $CD4^+8^-$ and $CD4^-8^+$ medullary T cells. Intrathymic injection as well as in vivo labeling experiments that monitored the fate of $CD4^+8^+$ cells lend support to this conclusion (Petrie et al., 1990; Shortman et al., 1991).

The high rate of death within the thymus appears to reflect the molding of the initial TCR repertoire. Mature T cells respond to antigen in the context of self major histocompatibility (MHC) molecules, yet must avoid high-affinity reactivity with self peptides. During differentiation in the thymus, the TCR repertoire is shaped by both positive and negative events involving MHC molecules (Blackman et al., 1990). Evidence for positive selection was first provided by bone marrow transplantation and thymus grafting experiments in which MHC molecules dictated the restriction specificity of developing T cells (Bevan, 1977; Zinkernagel et al., 1978). Most definitively, transgenic mice that express a single TCR indicate that the maturation of their T cells required a positive interaction between TCR and MHC molecules (Kisielow et al., 1988b; Sha et al., 1988; Berg et al., 1989; Scott et al., 1989). In contrast, cells expressing TCR Vβ regions that demonstrate high-affinity reactivity to self antigens are deleted during T cell development (Kappler et al., 1987). Elegant transgenic models that express a TCR specific for the male (HY) antigen, class I MHC, or peptide in the context of class II MHC have provided additional evidence for the negative selection of autoreactive T cells (Kisielow et al., 1988a; Sha et al., 1988; Murphy et al., 1990). Most immature thymocytes do not survive this selective gauntlet, and transgenic models argue that the eliminated cells die by apoptosis within the thymus (Murphy et al., 1990). While both forms of selection are dependent upon the specificity of the TCR, the precise molecular events that determine the positive or negative outcome remain uncertain.

The *bcl-2* gene was identified at the chromosomal breakpoint of t(14;18) bearing indolent B cell lymphomas (Tsujimoto et al., 1985; Bakhshi et al., 1985; Cleary and Sklar, 1985). *bcl-2* is novel among proto-oncogenes in that it localizes to the inner mitochondrial membrane (Hockenbery et al., 1990). *bcl-2* demonstrates the unique functional role of blocking programmed cell death in selected hematopoietic cell lines following cytokine deprivation (Vaux et al., 1988; Nunez et al., 1990; Hockenbery et al., 1990). Transgenic mice bearing a *bcl-2*–immunoglobulin minigene display extended B cell survival (McDonnell et al., 1989; McDonnell et al. 1990). Moreover, latent membrane protein 1 of Epstein–Barr virus has recently been shown to upregulate *bcl-2* and block apoptosis in Epstein–Barr virus–infected B cell lines (Henderson et al., 1991). bcl-2 protein is geographically restricted in tissues characterized by apoptotic cell death (Hockenbery et al., 1991). For example, bcl-2 is confined to the zones of surviving B cells in germinal centers. bcl-2 is also topographically restricted in the thymus where the vast majority of cortical thymocytes lack bcl-2, while the mature thymocytes in the medulla are positive (Pezzella et al., 1990; Hockenbery et al., 1991). This regional distribution suggests that *bcl-2* is differentially regulated during T cell maturation and involved in the salvation of T cells.

To assess the role of *bcl-2* in T cell development we expressed *bcl-2* in the immature T cells in the cortex of the thymus. We sought to determine the effect of placing this

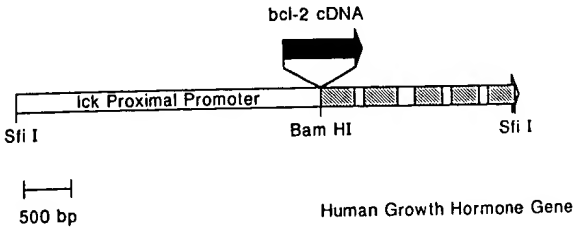

Figure 1. *lck*^{Pr}–*bcl*-2 Transgene Construct

A 0.75 kb cDNA of human *bcl*-2 (closed box) was inserted at the BamHI site 3′ to the 3.2 kb *lck*^{Pr} (open box). Introns and exons (hatched boxes) of *hGH* constitute the 3′ untranslated region.

particular antidote to programmed death afferent to thymic selection. The proximal promoter of the *lck* tyrosine kinase gene (*lck*^{Pr}) is expressed in immature cortical thymocytes (Reynolds et al., 1990; Wildin et al., 1991). A *lck*^{Pr}–*bcl*-2 transgene redirected bcl-2 to the normally vulnerable cortical thymocytes and protected them from a wide variety of apoptotic stimuli including glucocorticoids, radiation, and anti-CD3 treatment. Yet, clonal deletion of self-reactive T cells still occurred, suggesting that negative selection operates through a bcl-2-independent pathway.

Results

lck^{Pr}–*bcl*-2 Transgenic Mice

We developed a transgenic mouse model to assess the effects of bcl-2 upon T cell development. A transgenic construct was generated by inserting a human *bcl*-2 cDNA downstream of the *lck*^{Pr} (Chaffin et al., 1990; Garvin et al., 1990) (Figure 1). The 3′ untranslated portion of this construct provided introns, exons, and the poly(A) addition site from the human growth hormone gene *hGH*. Eight founder animals bearing the *lck*^{Pr}–*bcl*-2 construct were identified. Seven lines were established, and each line was examined for human bcl-2 expression in the thymus and spleen by Western immunoblot and immunohistochemistry. The three lines, 7, 17, and 36, with the highest levels of human bcl-2 were further characterized.

lck^{Pr} Redirects bcl-2 to the Thymic Cortex

The tissue specificity of the *lck*^{Pr}–*bcl*-2 transgene was examined by Western blot analysis, which revealed that bcl-2 was expressed only in lymphoid tissues (Figure 2A). Human bcl-2 protein was abundant in thymus and spleen but was not expressed in the large intestine, kidney, liver, heart, or lung. All transgenic lines demonstrated bcl-2 protein in the thymus, spleen, and lymph nodes (both mesenteric and axillary/inguinal lymph nodes) (Figure 2B). bcl-2 protein was higher in the thymus than in the spleen or lymph nodes, consistent with the percentage of T cells in these organs. This distribution may also reflect the fact that the *lck*^{Pr} is more active in immature thymocytes than mature thymocytes and peripheral T cells (Reynolds et al., 1990; Wildin et al., 1991).

Immunohistochemical analysis of *lck*^{Pr}–*bcl*-2 transgenic mice revealed bcl-2 expression in both cortical and medullary thymocytes (Figure 3). The 6C8 anti-human bcl-2

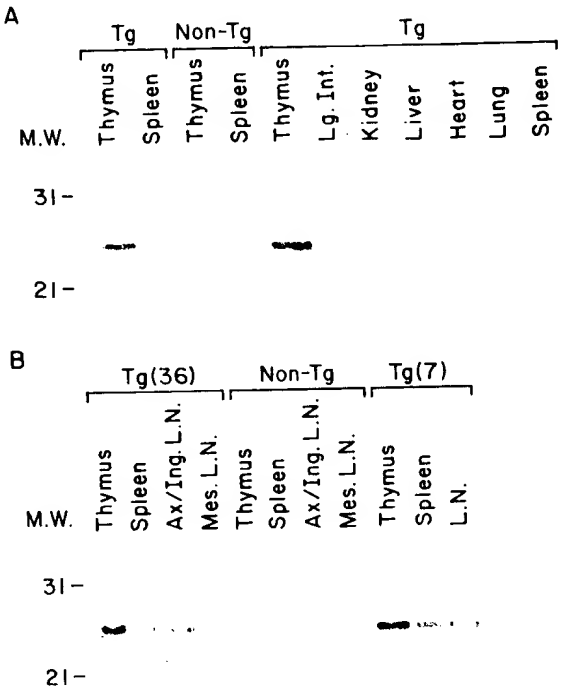

Figure 2. Analysis of Human *bcl*-2 Transgene Expression

Western blot analyses of cell lysates with the 6C8 mAb.

(A) Reactivity of cell lysates from transgenic line 36. Twenty-five micrograms of protein was loaded in each lane from the organ indicated.

(B) Expression of bcl-2 in lymphoid tissues. Twenty-five micrograms of protein from cell lysates from thymus, spleen, pooled axillary and inguinal lymph nodes, or mesenteric lymph nodes was analyzed. Tissues were isolated from transgenic lines 36 and 7, and nontransgenic littermates. Similar results were obtained from line 17.

monoclonal antibody (MAb) detected cytoplasmic bcl-2 in almost all lymphocytes in the thymus. In the spleen, there was prominent staining of cells in the white pulp with scattered positive cells in the red pulp. *lck*^{Pr}–*bcl*-2 mice between 4 and 10 weeks of age displayed normal architecture of all lymphoid organs. The thymus contained a distinct cortex and medulla and was normal in size. The distribution of splenic red and white pulp was similar in transgenic and control littermate mice.

Increased CD3^{hi} and CD4^{-}8^{+} Thymocytes

While expression of bcl-2 in the cortex did not substantially increase the number of thymocytes, it altered the distribution of T cell subsets. Transgenic and control mice possessed similar numbers of CD3^{-} thymocytes (~20%). All transgenic animals had an increased percentage of CD3^{hi}/TCR^{hi} thymocytes (~40%) compared with controls (~15%) (Figure 4). Reagents directed to TCR molecules revealed a parallel increase in αβTCR^{hi} thymocytes and no alteration in the percentage of γδ T cells (data not shown). Transgenics displayed a reciprocal decrease in CD3^{lo} cells (Figure 4). Thymocytes that have successfully completed thymic selection demonstrate increased CD3 expression, whereas CD3^{lo} cells represent immature T cells, most of

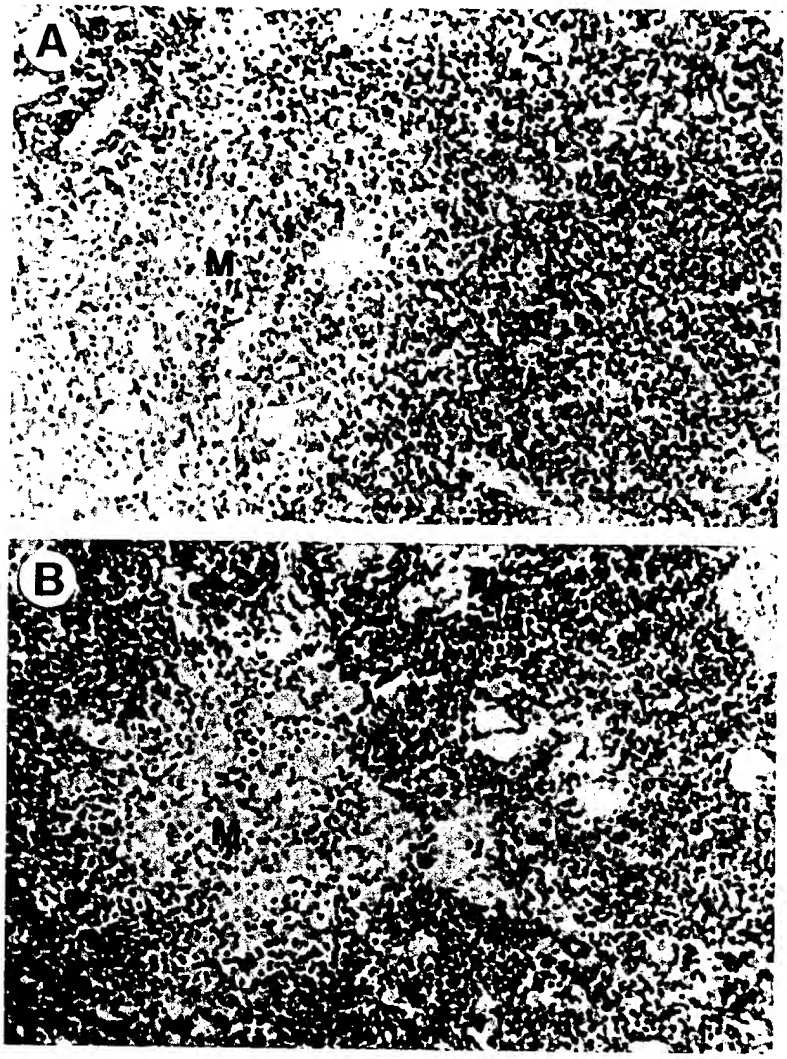

Figure 3. Immunohistologic Analysis of Human bcl-2 in Transgenic Thymus

Frozen serial sections were stained with (A) 6C8 MAb or (B) anti-human TNF, then all sections were counterstained with methyl green–Alcian blue. Medullary (M) and cortical (C) areas are indicated. Similar results were obtained with all 7 *lck*^{Pr}*–bcl*-2 transgenic lines. Control littermate animals demonstrated no reactivity with the 6C8 anti-human bcl-2 MAb (data not shown).

which are believed to die while undergoing thymic selection. It is of interest that many of the CD3^{hi} cells in transgenics could be classified as having an intermediate level of TCR and CD3. This TCR/CD3^{med} population has been proposed as a transitional intermediate stage following positive selection (Guidos et al., 1990; Shortman et al., 1991).

In addition, *lck*^{Pr}*–bcl*-2 transgenics uniformly demonstrated an increase in CD4$^-$8$^+$ single positive thymocytes (Figure 5). Some animals also displayed an increased percentage of CD4$^+$8$^-$ single positive cells. The increase in CD4$^-$8$^+$ thymocytes changed the average ratio of CD4$^+$8$^-$/CD4$^-$8$^+$ cells from 3.9 in control mice to 1.3 in transgenic mice. Immunoblot analysis revealed no difference in the amount of bcl-2 protein in CD4$^+$8$^-$ versus CD4$^-$8$^+$ cells (data not shown). In addition, transgenic mice possessed more of the CD4$^+$8^{lo} and CD4^{lo}8$^+$ subpopulations. This pattern of CD4 and CD8 expression is found on the CD3^{med} population that is thought to be an intermediate stage of thymocyte development (Guidos et al., 1990). In spite of the increase in mature thymocytes as measured by CD3, CD4, and CD8 parameters, no change was observed in MEL-14 or IL-2 receptor expression. The percentage of J11D$^-$ cells increased from 6% to 12%. Thus, the phenotypic effect of expressing bcl-2 in the cortex was an increase in mature thymocytes skewed towards the CD8$^+$ (class I restricted) subset.

Flow cytometric analysis of spleen and lymph nodes from transgenic mice revealed an increase in both the number of T cells (Thy1$^+$, CD3$^+$ cells) and the percentage of CD8$^+$ cells (Figure 5). Many of these were CD8^{lo}. The total number of mononuclear cells in transgenic spleens was increased from 40% to 100%. The percentage of CD8$^+$ T cells nearly doubled in the transgenic animals (22%–39%). The ratio of CD4$^+$/CD8$^+$ T cells in the lymph nodes of transgenic mice averaged 1.4 compared with 2.6 in control littermate mice. While the percentage of B cells in transgenic spleens was decreased by 40%, the absolute number of B cells was comparable in transgenic and control spleens.

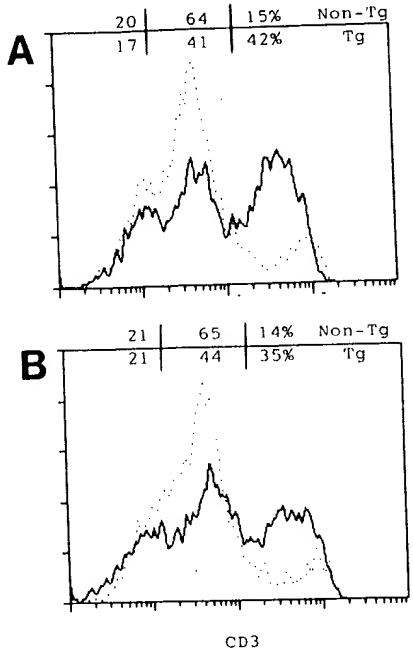

Figure 4. Expression of CD3 on the Thymocytes

Single-cell suspension of thymocytes from transgenic (solid lines) and nontransgenic (dotted lines) were stained with 2C11 MAb (Anti-CD3ε) followed by FITC-conjugated goat anti-hamster sera. Data from line 17(A) and line 36(B) are shown. The percentage of cells that are $CD3^-$, $CD3^{lo}$, or $CD3^{hi}$ are indicated above each histogram.

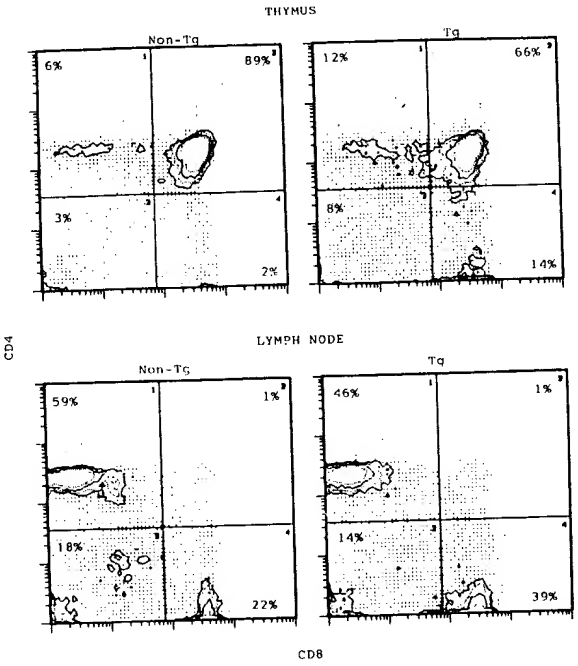

Figure 5. Two-Color Immunofluorescence Contour Plots of CD4 and CD8 Expression in Transgenic (Tg) and Nontransgenic (non-Tg) Mice

Representative histograms of thymocytes or lymph node cells stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8. The percentage of cells of each phenotype is indicated. Similar results have been obtained in all lines tested : 7, 17, 36, and 37.

Increased Survival of Transgenic Thymocytes

Immature $CD4^+8^+$ cortical thymocytes die rapidly in culture (Fowlkes and Pardoll, 1989). To evaluate the effects of bcl-2 on these cells, suspensions of thymocytes from *lck*^{Pr}–*bcl-2* and control littermates were placed in vitro in Iscove's minimal essential medium supplemented with 5% fetal calf serum (Figure 6). Thymocytes from transgenic and normal mice had a similar initial death rate, but after 24 hr transgenic thymocytes demonstrate improved survival. After 6 days, 30% of *lck*^{Pr}–*bcl-2* thymocytes were viable whereas <1% of the normal thymocytes survived. Flow cytometric analysis at day 6 revealed the persistence of all three subsets of thymocytes in transgenics: $CD4^+8^-$ (28%), $CD4^-8^+$ (24%), and $CD4^+8^+$ (48%). Similarly, splenic T cells also demonstrated increased survival in vitro. However, there were no detectable differences between transgenic and control thymocytes or spleen cells in their proliferative responses when cultured with Concanavalin A, MHC disparate cells, or medium alone (data not shown).

bcl-2 Blocks Glucocorticoid Depletion of Thymocytes

Since bcl-2 extended the survival of thymocytes in vitro, we questioned whether it might serve a generalized role of opposing apoptosis. Given the array of stimuli that can induce apoptosis in thymocytes, these transgenic mice provided a model to assess the effects of bcl-2 upon each pathway. Glucocorticoid treatment is known to cause a rapid depletion of cortical thymocytes by apoptosis both in vivo and in vitro (Blomgren and Andersson, 1971; Cohen and Duke, 1984). Preliminary experiments indicated that 0.1 mg of dexamethasone intraperitoneally depleted ~85% of thymocytes in (C3H × C57BL/6)F_1 control mice. In five independent experiments transgenic and nontransgenic control mice received dexamethasone, and thymocytes were evaluated 48 hr later (Figure 7A). Dexamethasone at 0.1 or 0.5 mg depleted a mean of 90% and 98% of thymocytes respectively in control mice, as compared with vehicle treatment alone. However, *lck*^{Pr}–*bcl-2* mice were markedly resistant with an average decrease of only 10%–20% with either dose. Flow cytometric analysis of the surviving thymocytes indicate that dexamethasone did not significantly alter the $CD4^+8^+$ thymocytes in transgenic mice, but almost completely eliminated the $CD4^+8^+$ population from control mice (Figure 7B). In addition, an in vitro system to evaluate apoptosis revealed that transgenic bcl-2 inhibited DNA fragmentation induced by 1 μM dexamethasone (Figure 8A).

bcl-2 Inhibits Radiation-Induced Cell Death

The in vitro system monitoring DNA fragmentation was used to assess the effects of bcl-2 upon radiation-induced apoptosis. Low-dose radiation (225 rads) resulted in fragmentation of 50% of the DNA in control thymocytes after 5 hr. However, transgenic thymocytes were protected from radiation (Figure 8B). Moreover, transgenic bcl-2 decreased the spontaneous DNA fragmentation of cultured thymocytes at 5 hr from 20% to 8% (Figures 8A and 8B).

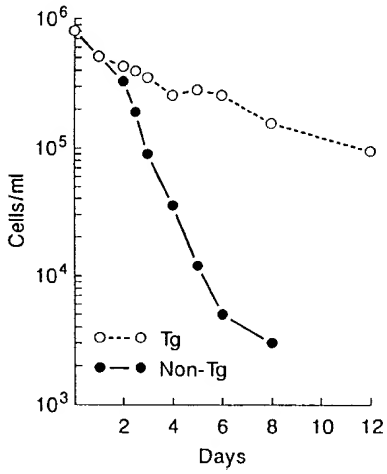

Figure 6. In Vitro Survival of Thymocytes

Total viable cells per ml recovered after in vitro culture with 5% FCS in Iscove's minimal essential medium. Cells were initially plated at 8 × 10^5 cells per ml in 96-well flat-bottomed plates. Each data point represents the mean value of triplicate cultures.

Lck^{Pr}*-bcl-2* Thymocytes are Resistant to Anti-CD3-Induced Apoptosis

bcl-2 can inhibit several forms of programmed death initiated by general stimuli. However, it was of interest to determine whether bcl-2 could block a T cell–specific apoptotic event. Triggering thymocytes with anti-CD3 has been shown to induce apoptosis both in vivo and in vitro (Smith et al., 1989; Shi et al., 1991). Consequently, mice were treated with anti-CD3, and thymi were examined 15 hr later. Following treatment, the number of $CD4^+8^+$ thymocytes was decreased substantially (85%–44%) in control mice, and their thymi demonstrated a classic nucleosomal-length DNA fragmentation pattern (Figure 9). *lck*^{Pr}*–bcl-2* mice were resistant to in vivo anti-CD3 treatment with no substantial change in thymocyte numbers or subsets and no evidence of DNA degradation. Moreover, the presence of transgenic bcl-2 blocked anti-CD3-induced depletion of $CD4^+8^+$ cells in an in vitro assay utilizing day 18 fetal thymic organ cultures (data not shown).

Transgenic bcl-2 Does Not Inhibit Negative Selection

One of the major mechanisms of tolerance to self antigen is the deletion of self-reactive T cells in the thymus. The presence of bcl-2 in the thymic cortex inhibited several apoptotic signals including anti-CD3 treatment, which has often been viewed as being analogous to clonal deletion. Therefore we asked whether *lck*^{Pr}*–bcl-2* transgenic mice would be unable to delete self-reactive T cells. T cells bearing certain Vβ regions are deleted in the thymus because of their interaction with specific self superantigen in the context of the MHC class II molecule I-E (Dyson et al., 1991; Frankel et al., 1991; Marrack et al., 1991; Woodland et al., 1991). T cells that express Vβ11, Vβ5, or Vβ17a are diminished in the periphery of mice that possess a specific endogenous superantigen in association with I-E (Kappler et al., 1987; Woodland et al., 1990). C57BL/6 mice (H-2^b) were crossed to transgenic mice that were heterozygous for their MHC (H-$2^{b/k}$). The H-$2^{b/b}$ offspring did not express I-E and possessed ~5% $V\beta11^+$ and ~4% $V\beta5^+$ $CD4^+$ T cells irrespective of their transgene status (Table 1). In contrast, the expression of I-E resulted in the elimination of most $V\beta11^+$ and $V\beta5^+$ T cells in both transgenics and control littermates. The usage of Vβ8 and Vβ6, which are not negatively selected by I-E/superantigen, was comparable in all mice.

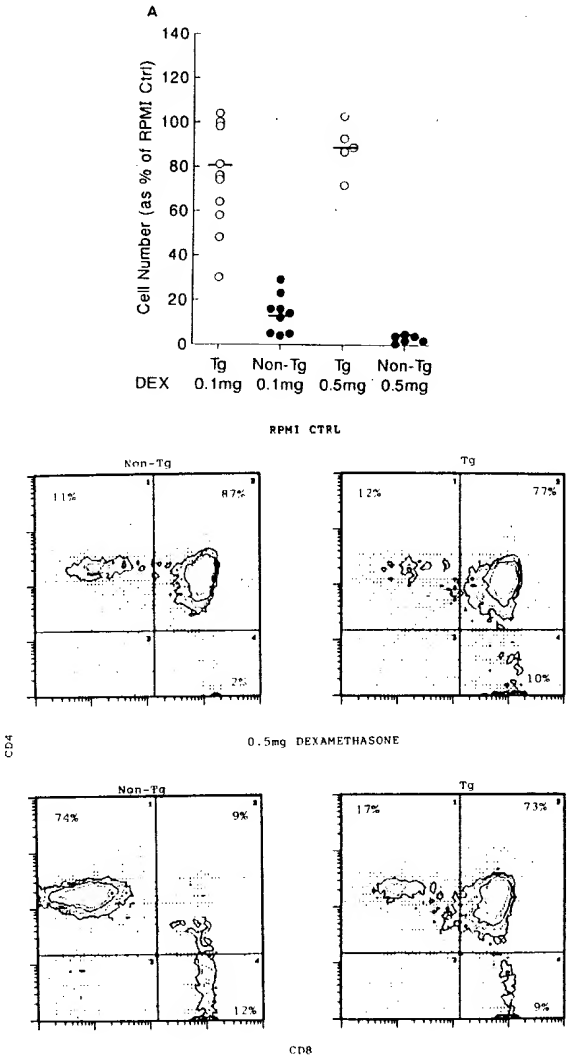

Figure 7. Dexamethasone Depletion of Thymocytes In Vivo

(A) Total thymocytes recovered 48 hr after in vivo treatment with 0.1 mg or 0.5 mg of dexamethasone intraperitoneally. Data represent a total of five independent experiments with animals from line 17 and 36. The animals ranged between 6 and 12 weeks of age but were identical in age within an experiment. Controls (100% values) of both transgenic and nontransgenic mice were treated with RPMI 1640 for each experiment. Total thymocytes recovered from RPMI 1640 treated animals ranged from 37–80 × 10^6 cells.

(B) Two-color immunofluorescence contour plots of CD4 and CD8 expression on surviving thymocytes. Thymocytes recovered from transgenic (Tg) and nontransgenic (non-Tg) mice 48 hr following treatment with RPMI 1640 or 0.5 mg of dexamethasone were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8. The percentage of cells of a given phenotype is indicated.

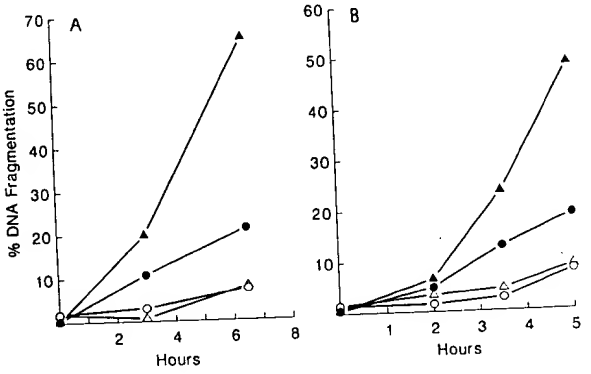

Figure 8. Percent DNA Fragmentation of Thymocytes

Thymocytes were incubated in vitro with (A) 1 μM dexamethasone (triangles) or (B) after 225 rad treatment (triangles). Cultured but untreated thymocytes were included (circles). Thymocytes from transgenic (open symbols) and nontransgenic (closed symbols) mice are shown. All data points represent the mean of triplicate cultures. Similar data were obtained with transgenic lines 7, 17, and 36.

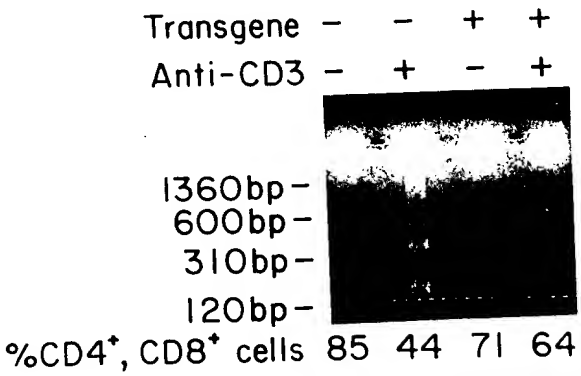

Figure 9. DNA Fragmentation of Thymocytes Following In Vivo Anti-CD3 Treatment

Two hundred fifty micrograms of anti-CD3 or vehicle control was injected into mice 14 hr prior to removal of the thymus. Each lane represents DNA from 1.5 × 10^6 thymocytes. The percentage of viable thymocytes that expressed CD4 and CD8 from each animal is indicated.

To examine the deletion of Vβ17a$^+$ T cells, transgenic mice were crossed to SJL (H-2^s) mice that possess a Vβ17a gene. Transgenic and nontransgenic mice that did not express I-E (H-$2^{s/b}$) demonstrated 3.6% and 3.3% Vβ17a$^+$ CD4$^+$ T cells respectively. In the presence of I-E (H-$2^{s/k}$), transgenics (0.7%) as well as nontransgenics (0.5%) deleted Vβ17a$^+$ cells (Table 1). The Vβ11$^+$ CD4$^+$ T cells were also deleted in H-$2^{s/k}$ mice irrespective of transgene status. Since Vβ11, Vβ5, and Vβ17a TCRs are all deleted in the context of class II MHC, Table 1 presents the data for CD4$^+$ T cells. However, total peripheral T cells were similarly affected.

Deletion of self-reactive T cells occurs in the thymus and is evident by the stage of mature CD3^{hi} single positive thymocytes. The percentage of Vβ11^{lo} and Vβ17a^{lo} thymocytes was similar in all mice arguing that *lck*^{Pr}–*bcl*-2 transgenics produce comparable numbers of cells bearing these TCRs. Transgenic and nontransgenic mice that express I-E had comparably deleted Vβ11^{hi} and Vβ17a^{hi} thymocytes (Table 2). Thus, the lack of self-reactive T cells in the periphery of *lck*^{Pr}–*bcl*-2 transgenic mice reflects intrathymic clonal deletion.

Discussion

bcl-2 belongs to a novel category of oncogenes in that it has been shown to block certain forms of cell death rather than promote proliferation. Overexpressed bcl-2 prevented the apoptotic death of selected hematopoietic cell lines following deprivation of IL-3, IL-4, or GM-CSF; yet, it did not prevent the demise of IL-2- or IL-6-deprived cell

Table 1. Percentage of CD4$^+$ Lymph Node Cells

	C57BL/6(b) × Lck^{Pr}–Bcl-2 (b/k) Mice						SJL(s) × Lck^{Pr}–Bcl-2 (b/k) Mice				
Transgene	H-2	I-E	Vβ8	Vβ6	Vβ11	Vβ5	H-2	I-E	Vβ6	Vβ11	Vβ17a
–	b/b	–	23.7 (1.3)	8.3 (0.8)	4.8 (0.9)	4.1 (0.6)	s/b	–	9.3 (1.6)	2.9 (0.5)	3.3 (0.6)
+	b/b	–	22.2 (0.8)	9.1 (0.3)	5.6 (0.2)	3.8 (0.8)	s/b	–	9.4 (1.0)	3.2 (0.6)	3.6 (1.0)
–	b/k	+	29.0 (1.1)	9.2 (0.5)	0.7 (0.2)	0.5 (0.4)	s/k	+	10.1 (2.0)	0.8 (0.2)	0.5 (0.2)
+	b/k	+	26.2 (1.6)	9.2 (0.6)	2.1 (0.4)	0.6 (0.2)	s/k	+	9.5 (1.0)	1.3 (0.2)	0.7 (0.5)

Numbers represent the mean percentage of CD4$^+$ lymph node cells bearing each Vβ as determined by two-color flow cytometry. Standard deviations are shown in parentheses. Data represent at least three independent animals using offspring from lines 17 and 36.

Table 2. Percentage of Thymocytes in SJL(s) × Lck^{Pr}–bcl-2(b/k) Mice

			Percentage of CD3^{lo} Cells		Percentage of CD3^{hi} Cells	
Transgene	H-2	I-E	Vβ11^{lo}	Vβ17a^{lo}	Vβ11^{hi}	Vβ17a^{hi}
–	s/b	–	3.1 (1.2)	4.6 (3.4)	2.5 (0.2)	4.6 (1.8)
+	s/b	–	2.5 (0.7)	2.1 (1.5)	2.5 (0.1)	3.1 (1.2)
–	s/k	+	2.2 (0.2)	1.6 (1.0)	1.1 (0.4)	0.6 (0.4)
+	s/k	+	3.2 (0.3)	2.1 (1.5)	1.3 (0.4)	1.0 (0.2)

The numbers represent the mean percentage of CD3^{lo} or CD3^{hi} thymocytes bearing each Vβ as determined by two-color flow cytometry. Standard deviations are shown in parentheses.

lines (Vaux et al., 1988; Nunez et al., 1990; Hockenbery et al., 1990). In vivo, a deregulated *bcl-2*–immunoglobulin transgene enhanced the survival of resting B cells, prolonged B cell memory, and promoted high-grade malignancy (McDonnell et al., 1989; Nunez et al., 1991; McDonnell and Korsmeyer, 1991;). However, this transgenic model did not alter T cell survival. Within normal thymus bcl-2 is expressed in only several percent of cortical thymocytes, but is uniformly present in the medulla. This topographic distribution suggests that bcl-2 also has a prominent role in a T cell survival pathway.

The *lck*^{Pr}–*bcl-2* transgenic mice have substantially broadened the contexts in which bcl-2 regulates programmed cell death. In contrast to the *bcl-2*–immunoglobulin transgene, the *lck*^{Pr} successfully expressed bcl-2 in the CD4$^+$8$^+$ immature thymocytes. bcl-2 protected this population from a variety of stimuli that initiate cell death by different molecular signals. The elimination of cortical thymocytes following glucocorticoid treatment results from an apoptotic death program shown to be dependent upon de novo RNA and protein synthesis (Cohen and Duke, 1984). bcl-2 effectively blocked even high-dose dexamethasone-induced thymocyte death. Glucocorticoids bind to cytosolic receptors that are translocated to the nucleus where the complex interacts with glucocorticoid response elements resulting in gene transcription. A number of points exist within this pathway where a regulator of cell death might interfere.

Low-dose irradiation induces cell death in resting thymocytes and results in an arrest in the G2 phase of the cell cycle prior to mitosis (Sellins and Cohen, 1987; Lucke-Huhle et al., 1979). Radiation results in the generation of free radicals, which are felt to induce oxidative base damage resulting in DNA strand breaks and the production of thymine glycols. Overproduced bcl-2 prevents the death of resting thymocytes following exposure to 225 rads. This raises the possibility that bcl-2 represents the molecular mediator of a number of radioprotective agents that have been described (Neta et al., 1988).

Treatment of thymocytes with anti-CD3 results in a lineage-specific apoptosis. Signal transduction events following anti-CD3 treatment include a sustained increase in cytosolic Ca^{2+} and phosphoinositol turnover (Altman et al., 1990). bcl-2 blocked anti-CD3-induced apoptosis in immature thymocytes. Thus, bcl-2 is capable of blocking programmed cell death following different stimuli. Moreover, bcl-2 is topographically restricted to the progenitor cells and long-lived postmitotic cells in a number of tissues all characterized by apoptotic cell turnover (Hockenbery et al., 1991). The *lck*^{Pr}–*bcl-2* mice suggest that bcl-2 acts at a common decisional checkpoint in cell death that is distal to the initial signalling events.

While transgenic bcl-2 could block the induction of apoptosis by multiple stimuli, the negative selection of reactive T cells against superantigens remained intact. The vast majority of Vβ5, Vβ11, and Vβ17a$^+$ T cells was still deleted in mice that expressed I-E despite the presence of the *bcl-2* transgene. The slight increment in Vβ11$^+$ cells in transgenics (2.1 ± 0.4%) versus controls (0.7 ± 0.2%) that expressed H-2$^{b/k}$ might conceivably reflect the escape of low-affinity self-reactive Vβ11 cells. However, this is not a consistent phenomenon as Vβ5$^+$ and Vβ17a$^+$ T cells were essentially identical in transgenics and controls. In addition the H-2$^{s/k}$ background displayed no differences in Vβ11$^+$ cells (Table 1). Negative selection occurred in the thymus in mice that expressed I-E regardless of the presence of the *bcl-2* transgene (Table 2). Anti-CD3 treatment results in activation-induced cell death (Finkel et al., 1989; Shi et al.,1991; Smith et al., 1989) and has been proposed to reflect an in vitro model of negative selection. However, the *lck*^{Pr}–*bcl-2* mice clearly indicate that soluble anti-CD3 treatment is not the experimental equivalent of negative selection. Accessory molecules including CD4 and CD8 are crucial to T cell development (Robey et al., 1991; Fung-Leung et al., 1991), and evidence suggests that other molecules may also participate (Turka et al., 1991; Ashwell and Klausner, 1990). It is intriguing to speculate that negative selection requires the interaction of accessory molecules as well as the engagement of T cell receptors. It is formally possible that high-affinity anti-self reactivity results in a signal intensity that overwhelms the capacity of transgenic bcl-2 to block apoptosis. However, the level of deregulated bcl-2 was sufficient to block death by both high-dose glucocorticoid and radiation, which are not weak stimuli. Such evidence argues that negative selection represents a bcl-2-independent pathway.

The demarcation in normal thymus in which CD4$^-$8$^+$ and CD4$^+$8$^-$ medullary thymocytes uniformly express bcl-2 raises the possibility that bcl-2 might only be expressed after completion of thymic selection. If the role of bcl-2 was only to maintain mature thymocytes and T cells, it would not be expected to alter thymic maturation. Instead, *lck*^{Pr}–*bcl-2* mice displayed a marked aberration in T cell development with an increase in CD3^{hi} thymocytes. During normal T cell selection, cells that recognize self MHC but lack high-affinity reactivity to self peptides survive and, as a result, increase their CD3 expression and lose either CD4 or CD8 expression. The reciprocal decrease in CD3^{lo} cells in transgenic mice suggests that bcl-2 allows the maturation of cells that would normally die from a lack of positive selection. This prediction provides a testable hypothesis to determine whether bcl-2 promotes the selection of T cells without self MHC restriction.

These transgenic mice have distinguished T cell death programs that are either bcl-2-dependent or -independent. Studies of T cell hybridomas have also suggested the existence of separate cell activation and steroid-induced death programs (Zacharchuk et al., 1990). *lck*^{Pr}–*bcl-2* transgenic mice provide a model to elucidate further the biochemical basis of the selective interference of bcl-2 with cell death programs.

Experimental Procedures

Animals

All animals were bred and maintained in a pathogen-free environment at Washington University Medical School. C3H/He Swiss (C3H × C57BL/6)F$_1$ mice were purchased from Taconic Lab Animals and Services (Germantown, NY) and C57BL/6 from Jackson Laboratory (Bar Harbor, ME).

Construction of *lck*^{Pr}-*bcl*-2

The construct was generated by insertion of a 0.75 kb fragment containing the coding region of human *bcl*-2 cDNA #58 (Seto et al., 1988) into the *lck-hGH* vector (Chaffin et al., 1990). The *bcl*-2 cDNA was inserted by blunt-end ligation into the BamHI cloning site 3′ to the *lck*^{Pr}. Correct orientation was selected, and SfiI was used to prepare the 6.3 kb *lck*^{Pr}-*bcl*-2 used for microinjection (Figure 1).

Production of Transgenic Mice

Transgenic mice were produced as previously described (McDonnell et al., 1989).

Tissue Immunohistochemistry

Tissue was isolated from transgenic or littermate controls and treated as frozen sections. Serial sections were fixed in acetone at 4°C for 10 min and treated with 0.18% H_2O_2 in methanol for 30 min. The sections were blocked with avidin, followed by biotin (Vector), and then blocked with 1% goat serum for 30 min. The 6C8 hamster anti-human bcl-2 MAb or a control hamster anti-TNF MAb, adjusted to equal concentrations, was incubated on the sections for 1 hr, followed by biotinylated horse anti-hamster IgG (Vector) at a 1:40 dilution, and ABC reagent (Vector) for 45 min each. The sections were counterstained with methyl green–Alcian blue, dehydrated, and mounted.

Western Blot Analysis

Single-cell suspensions were lysed in 150 mM NaCl, 10 mM Tris (pH 7.4), 1% triton X-100 with 2 μg of aprotinin ml^{-1} for 30 min at 4°C. After centrifugation at 27,000 × g, the amount of protein in the supernatants was quantitated using the method of Bradford (1976). The subsequent procedure has been described (McDonnell et al., 1990).

Flow Cytometry Reagents and Antibodies

Flow cytometry reagents were as follows: fluorescein isothiocyanate (FITC)-conjugated anti-murine CD4 and anti-murine CD8, phycoerytherin (PE)-conjugated anti-murine CD4 (Pharmingen, San Diego, CA); PE-conjugated streptavidin (Biomeda); anti-murine CD3ε (2C11, gift from Robert Schreiber, Deptartment of Pathology, Washington University); PE-conjugated anti-murine H-2K^b and FITC-conjugated anti-murine H-2K^k (Pharmingen); FITC-conjugated goat anti-hamster IgG (Caltag). Anti-T cell receptor antibodies were as follows: biotin-conjugated anti-Vβ6 (RR4-7), anti-Vβ8 (F23.1), anti-Vβ11 (RR3-15), anti-Vβ5 (94); anti-Vβ17a (KJ23a, gift from Judith Kapp, Dept. Pathology, Washington University).

Cell Surface Analysis

For flow cytometry, cells were washed twice in staining buffer, 1% fraction 5 bovine serum albumin (Sigma Chemical Co., St. Louis) in phosphate-buffered saline. Each sample of 10^6 cells was stained in 100 μl of buffer. Primary incubation was with staining buffer alone (negative control) or with 1 μg of specific antibody for 30 min at 4°C followed by washing with staining buffer. In the secondary incubation, all samples were stained with PE- or FITC-conjugated specific antibodies (anti-CD4 or anti-CD8), with PE- or FITC-conjugated streptavidin, or with the appropriate species-specific secondary antibodies for 30 min at 4°C. After additional washes in staining buffer, the samples were resuspended in 0.4 ml of staining buffer and analyzed on a FACSCAN analyzer (Becton-Dickinson, Mountain View, CA).

Percent DNA Fragmentation

Thymocytes (2×10^6) were washed and lysed in 0.5 ml of extraction buffer (5 mM Tris [pH 8.0], 20 mM EDTA, 0.5% Triton X-100) for 30 min at 4°C. The chromatin was separated from fragmented DNA by centrifugation at 27,000 × g for 30 min at 4°C. The supernatant was removed, and the pellet resuspended in 0.5 ml of extraction buffer. DNA from both pellet and supernatant was precipitated by the addition of 0.5 ml of 1 N perchloric acid. After centrifugation at 27,000 × g for 15 min, the supernatant was discarded, and 0.5 ml of 0.5 N perchloric acid was added. The DNA was hydrolyzed by incubation at 70°C for 20 min. The amount of DNA was quantitated by the diphenylamine method (Burton, 1956). Percent fragmentation refers to the ratio of DNA in the supernatant to the total DNA recovered in the supernatant plus pellet. Data are represented as the mean of 3 samples per data point.

Anti-CD3-Induced DNA Fragmentation

Two hundred fifty micrograms of affinity-purified anti-CD3 or vehicle control was injected into 7-week-old transgenic and control littermates. Thymi were removed 15 hr after treatment, and cell suspensions made by teasing in ice-cold RPMI 1640. Cells (3×10^6) were suspended in 0.5 ml of lysis buffer (10 mM EDTA, 50 mM Tris [pH 8.0], 0.5% Triton X-100) with 0.5 mg of proteinase K and incubated for 1.5 hr at 50°C. The samples were extracted three times with phenol-chloroform and precipitated with ethanol. After drying, DNA was resuspended in 50 μl of TE (10 mM Tris [pH 8.0], 0.1 mM EDTA) with 0.2 mg of RNAase A for 1 hr at 37°C. Half of each sample was mixed with 0.25% bromophenol blue and 40% sucrose prior to loading into the wells of a 2% agarose gel containing 0.05 μg of ml^{-1} ethidium bromide.

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